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ORIGINAL RESEARCH

Complexation of α-Mangostin with γ-Cyclodextrin and Its Application in Alginate/Chitosan Hydrogel Mucoadhesive Film for Treatment of Recurrent Aphthous Stomatitis

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Introduction: α-Mangostin (α-M), one of the xanthon compound isolated from *Garcinia mangostana* rind, demonstrates the efficacy in the treatment of recurrent aphthous stomatitis (RAS). The lack of solubility of α-mangostin in water limited its pharmacological application.

Purpose: The lack of solubility of α-mangostin in water limited its formulation and pharmacological application. This study was done to enhance the solubility of α-M by complexation with γ-cyclodextrin (γ-CD) and its application in Alginate/Chitosan Hydrogel Mucoadhesive Film (HMF) for RAS treatment.

Methods: This complex was made by dissolved α-M and γ-CD in separated solution. α-M solution gradualy added into γ-CD to formed α-M/γ-CD complex (α-M/γ-CD CX). This complex then evaporated to yield the dry complex powder. The complex was successfully formulated into hydrogel mucoadhesive film (HMF) preparations based on characterization using Scanning Electron Microscope (SEM), Fourier Transform Infra-Red (FTIR), and X-Ray Diffractometry (XRD). The complex was formulated in hydrogel mucoadhesive film, followed by in-vitro drug release and the study of recurrent aphthous stomatitis (RAS) activity in rats.

Results: The α-M/γ-CD CX HMF film has a higher mucoadhesive force and mucoadhesive time than other HMFs resulting in a prolonged retention time in the oral mucosa. The drug release of α-M/γ-CD CX HMF followed the Korsmeyer-Peppas Model with a total amount of drug released 80.34+0.32%. The inclusion complex of α-M/γ-CD CX HMF exhibited increased anti-RAS activity compared to HMF base, α-M HMF, and α-M/γ-CD PM HMF. This was evidenced by a significant decrease in wound area of approximately 79.05±3.30%, an increase in epithelial thickness of about 1.24±0.09 µm, and a decrease in neutrophil score 1.10±0.26. These findings highlight the potential use of α-M/γ-CD CX as an effective RAS agent in HMF.

Conclusion: The complex of α-M/γ-CD CX has improved solubility of α-M, resulting in the transparent and homogenous film. The film containing this complex has the better physical characteristic, increasing the release and RAS activity.

Keywords: α-Mangostin, γ-cyclodextrin, inclusion complex, solubility, hydrogel mucoadhesive film, recurrent aphthous stomatitis

Introduction

α-Mangostin (α-M)] is a xanthon that found in *Garcinia mangostana* pericarp extract.[1] α-M has been used as widely used as an anti-cancer, anti-fungal, antibacterial, anti-inflammatory, and antioxidant.[2–6] The latest, α-M and found to be effective for recurrent aphthous stomatitis.[7] The solubility of α-M in water become a barrier to apply α-M in several preparation. Many of methods were developed to improve solubility of α-M in water, such as reduction particles size to

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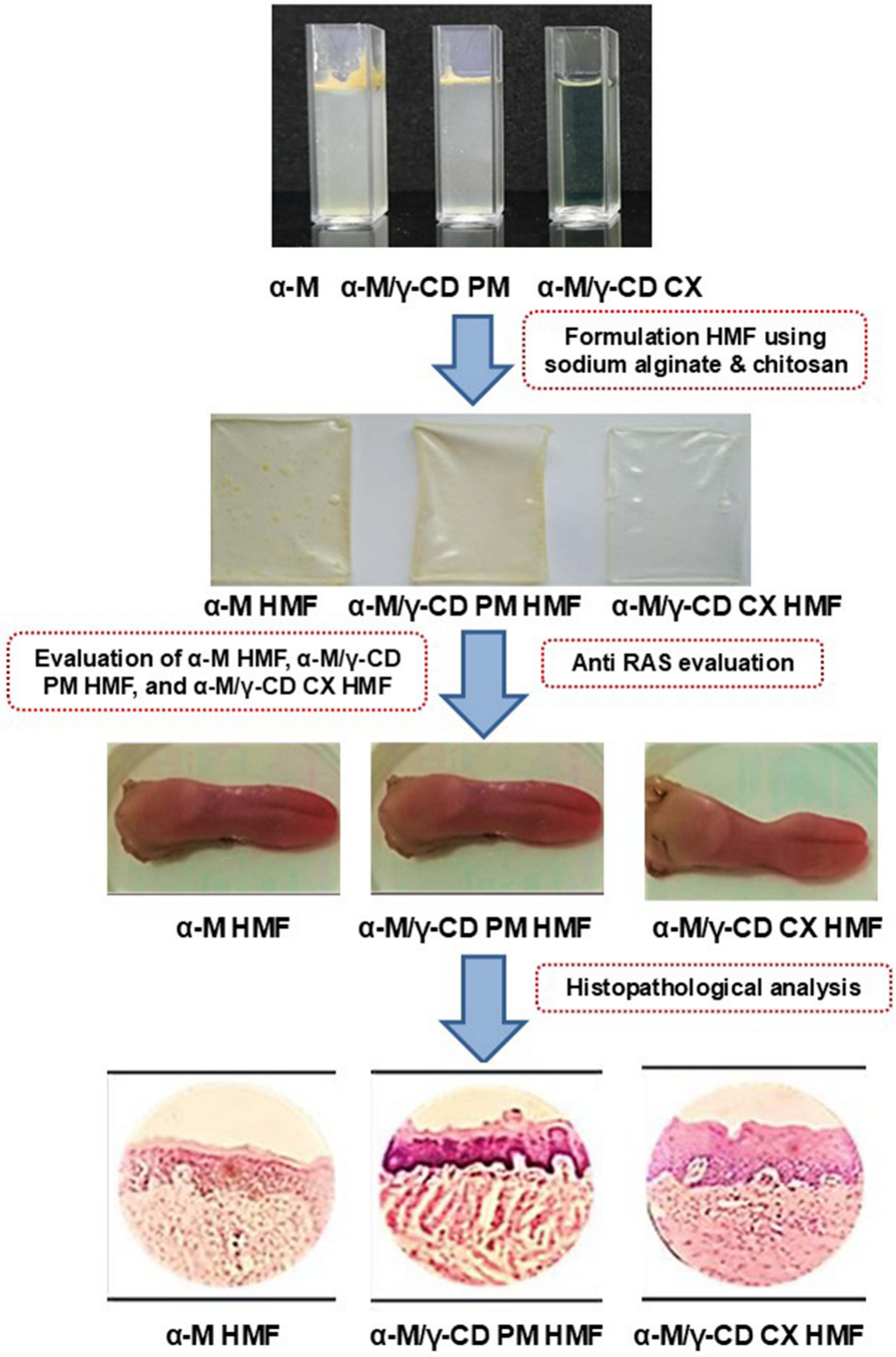
Graphical Abstract



increase the surface area, or complexation, making prodrugs, the use of certain polymers to modify drug delivery.[1,8–11] Nano micelles of α-M enhanced the solubility of α-M in 2743±11 μg/mL.[8] While the addition of ethanol rised the affinity of α-M to the β-CD molecule in complex formation.[10] The complexation of α−M with β-CD and 2,6-dimethyl-β-CD

rised the permeation.[12] γ-Cyclodextrin (CD) has the largest cavity from the other cyclodextrin, made from starch by enzymatic conversion, and has a conical truncated structure that is capable to entrap the hydrophobic molecule.[13,14] We have previously reported that hydroxypropyl-β-CD complex has the higher solubility in water and more effective in wound healing.[15] Recently, α-M has been reported to form an inclusion complex with γ-CD resulting in solubility enhancement.[13] The inclusion complex of γ-CD with forchlorfenuron were formed and increasing their water solubility.[16]

RAS is a common condition affecting the oral mucosa, including areas such as buccal mucosal layer, tongue and gums.[17] The prevalence of RAS in England ranges from 5% to 60%,[18] and globally it affects about 2% to 50% with the majority of lesions (83%) occurring in the mucosa.[19–21] RAS is characterized by the reddish to white lesions in the oral cavity. There are three type of RAS, -major, minor, and herpetiform-, classified based on size of the lesion.[22] Inhibiting the inflammatory response and promoting epithelial cell regeneration are the main goals of RAS treatment. Commonly used for treating RAS treatment include chlorhexidine gluconate, mometasone furoate, hyaluronic acid, cyclosporin, basic fibroblast growth factor (bFGF), Clobetasol propionate, dexamethasone, benzydamine, triamcinolone, and amlexanox.[23–28] The most frequently prescribed medications for RAS therapy are corticosteroids, which have a number of adverse effects on the oral mucosa, including mucosal pigmentation. Additionally, corticosteroids will cause a persistent infection and cutaneous bacterial complications. Corticosteroids play a role in inhibiting the inflammatory responses, with no effect on epithelial cell regeneration, making them less effective in the management of RAS.[27] Given the limitations of corticosteroid therapy for RAS, there is a need to develop more effective and efficient treatment options with fewer side effects. Therapy based on natural ingredients is an alternative to corticosteroids. Not only synthetic drugs were investigated for RAS treatment, but also several plants were used in their research to find the natural sources for RAS, for example *Punica granatum, Aloe vera, Myrtus communis*, licorice, *Ginger officinale, Pudilan xiaoyan*, and *Kouchang xiaoting*.[23,29–33] Research has shown that the active xanthone metabolite α-M found in mangosteen (*Garcinia mangostana*, L). has pharmacological efficacy in wound healing, suppressing inflammatory reactions, and promoting regeneration of epithelial cells.[34]

The preparation of hydrogel mucoadhesive film (HMF) is designed to extend the residence time of drugs on the mucosa. Alginate known for its ability to retain water, ion exchange, biocompatibility, and low toxicity. Its solubility in water is unstable, but it can form stable structures through gelation with multivalent cations, such as chitosan, which has non-toxic, anti-inflammatory, antimicrobial properties, tissue regeneration ability, is biodegradable, virucidal, fungicidal, and mucoadhesive, due to its ability to form films.[35–39]

In this study, the solubility enhancement was done by inclusion complex formation of α-M and γ-CD.[13] The resulting α-M/γ-CD complex (α-M/γ-CD CX) was incorporated into alginate-chitosan hydrogel mucoadhesive film (HMF) and evaluated for its study of anti-recurrent aphthous stomatitis (RAS) activity using animal models (Figure 1). To optimize the delivery of α-M, it was modified into a complex with γ-CD (α-M/γ-CD CX) and incorporated into an alginate-chitosan hydrogel mucoadhesive film (HMF). The effectiveness of HMF containing α-M/γ-CD CX was evaluated on animal models of RAS.

Materials and Methods

Materials

The materials used in study are α-Mangostin/α-M (Chengdu Pharmaceutical Industries, China), γ –Cyclodextrin/γ-CD (Kumamoto, Japan). Chitosan (Chi) and high purity deionized water (PT. Merck, Indonesia). Sodium alginate/Alg Wako® (Fujifilm Wako Pure Chemical Corporation, Japan), ethanol 95%, and glycerine (PT. Merck, Indonesia). The simulated saliva (SS) media pH 6.8 contains sodium chloride (PT. Merck, Indonesia), potassium phosphate monobasic (PT. Merck, Indonesia), sodium phosphate dibasic (PT. Merck, Indonesia). All materials were of analytical grade and were used without any further purification.

Preparation of α-Mangostin and γ-Cyclodextrin Complex (α-M/γ-CD CX)

α-M/γ-CD CX was prepared by the previously described method. 1 mm of α-M solution (in ethanol 95%) was added gradually into 2 mm of γ-CD solution (in water), then stirred for about 24 h at room temperature. The mixture was filtrated using 0.45 μm membrane, and the filtrate was evaporated at 60°C to yield the complex powder of α-M/γ-CD CX.[13]

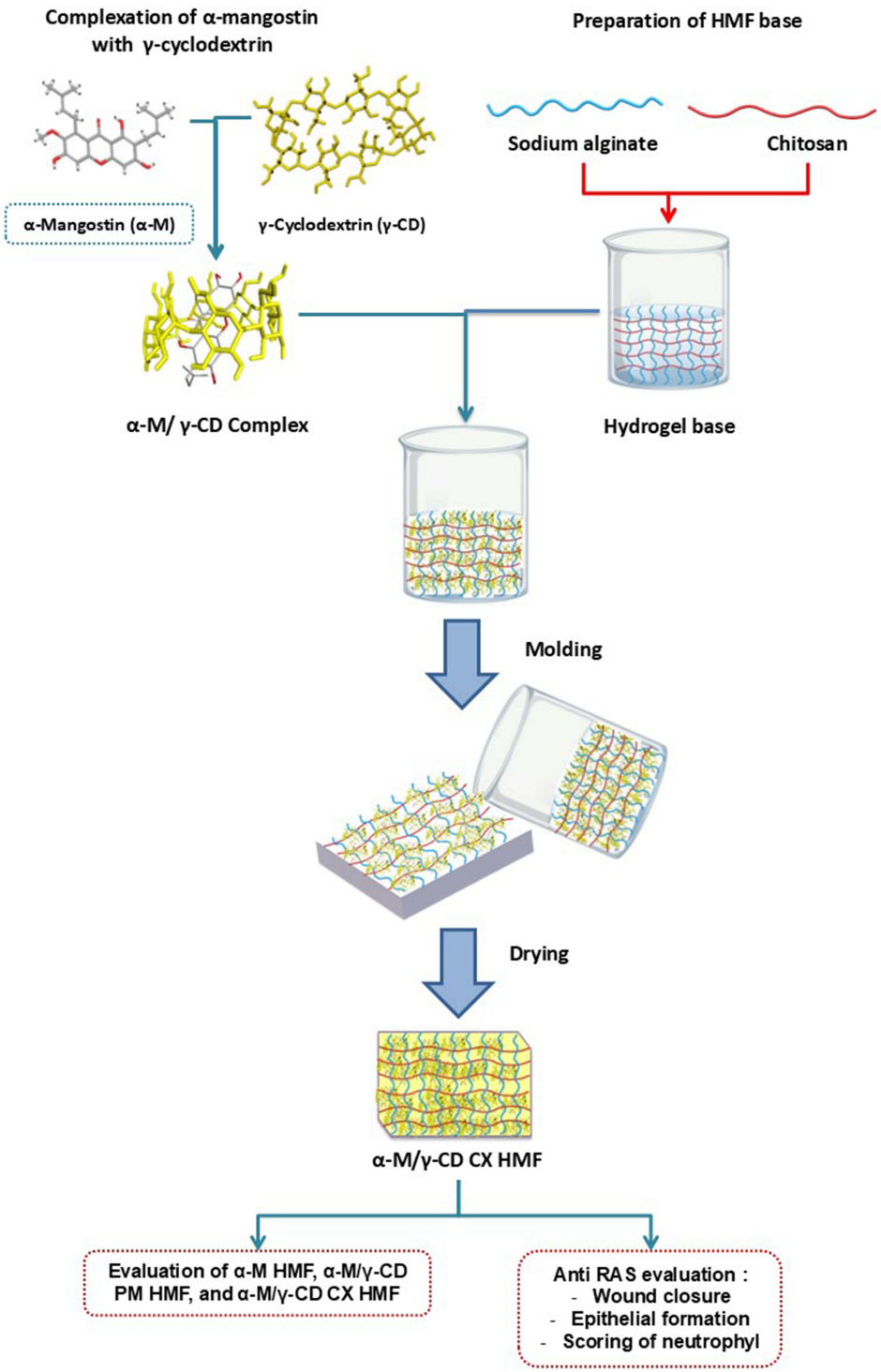


Figure 1 Design of experiment.

Preparation of Physical Mixture of α-Mangostin and γ-Cyclodextrin (α-M/γ-CD PM)

α-M/γ-CD PM was prepared by mixed α-mangostin powder with γ-cyclodextrin powder in ratio 1:2. This powder was placed into a vial and shaken for 1 minute by using vortex mixer to obtain physical mixture of α-mangostin with γ-cyclodextrin (α-M/γ-CD PM).[40]

Preparation of Hydrogel Mucoadhesive Film Base (HMF), α-M HMF, α-M/γ-CD PM HMF and α-M/γ-CD CX HMF

To prepare hydrogel mucoadhesive film base, sodium alginate was swelled in warm water, while chitosan was diluted in 1% v/v acetic acid. Chitosan was added gradually into the swelled alginate, and mixed until homogenous solution was achieved. Glycerine was added into the mixture and stirred until uniformly distributed (Table 1). The resulting mixture was then poured into a propylene box with dimensions of 7x5x2 cm^3 and 2–4 mm in height and dried at 60°C for 24 hours in oven to get a hydrogel mucoadhesive film.[7,41]

The α-M HMF was prepared by dissolving α-M powder in ethanol, which was then mixed with HMF base. α-M/γ-CD PM HMF was prepared by dissolving α-M/γ-CD PM powder in ethanol, then mixed with HMF base. Meanwhile, the α-M/γ-CD CX HMF was prepared by dissolving the complex in HMF base before the drying process. When the film was formed, the film was removed and stored in a closed place at room temperature.[41–43]

Physical and Mechanical Characteristics

The film thickness was measured by using Digital handled micrometer (Syntek, China), and the pH was examined by using FE20 pH-meter (Mettler Toledo).

Tensile strength and elongation at break (E%) of the film were measured by using extensometer (INSTRON 5943, United States). The film was clamped, and the tool was turned on at a speed of 2 mm/s. Measurements were taken five times. Tensile strength (TS) and elongation at break (E%) were calculated using the following equation:[41,44,45]

$$TS\ (MPa) = F\ x \frac{100}{t\ xW} \tag{1}$$

$$E\ (\%) = \frac{(L_1 - L_0)}{L_0} x100 \tag{2}$$

Tensile Strength (TS) is the maximum pressure required to break the film (N/cm^2). F is the maximum tensile force when the film breaks (N), t is the film thickness (mm), and W is the width of film (mm). L is the film length after drawing (mm), and L_o is the initial length (mm).

Swelling Index

Swelling index was evaluated by weighing 3×2 cm film, and soaking it in phosphate buffered saline (PBS) at pH 6.8 at 37°C. The completely expanded hydrogel film sheet was then removed. Excess PBS on the film was gently removed, and the film was weighed again (W_t). Swelling ratio was calculated using the equation:[44,46,47]

$$S\ (\%) = \frac{W_t - W_0}{W_0} x100 \tag{3}$$

W_o is initial weight of the film (g) and W_t is the weight of the film after swelling (g)

Table 1 Preparation of α-M/γ-CD HMF

Preparation	Alginate Sodium (g)	Chitosan (g)	Glycerin (%w/v)	α-M (μg)	Acetic Acid (mL)	Distilled Water (mL)
HMF	0.5	0.1	0.1	-	5	45
α-M HMF	0.5	0.1	0.1	250	5	45
α-M/γ-CD PM HMF	0.5	0.1	0.1	250	5	45
α-M/γ-CD CX HMF	0.5	0.1	0.1	250	5	45

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Water Vapour Transmission Rate (WVTR)

WVTR was measured using the drying method. The film was placed at the mouth of the vial containing 20.0 gram of activated silica gel.[48] Measurements were made at room temperature 30 C and relative humidity/RH 80%. Vial's weight was recorded every hour for 24 hours. WVTR is calculated by the following equation:

$$\text{WVTR}\left(\frac{\text{g}}{\text{hour.m}^2}\right)=\frac{(\text{W}_\text{t}-\text{W}_0)}{(\text{t xA})} \quad (4)$$

W_0 is the initial weight of HMF (g), W_t is the weight of HMF at the end of the evaluation, and A is permeation area (m^2).

Scanning Electron Microscopy (SEM)

To analyze the surface of the film, SEM analysis was carried out. A small piece of α-M HMF, α-M/γ-CD PM HMF, and α-M/γ-CD CX HMF were placed on aluminium strip and then coated with gold for 10 seconds (30 mm, 8 Pa). The surface of film was observed using SEM (JEOL JSM-6510LA Series, Tokyo, Japan) at a magnification of 5000x.[41,44,49]

Fourier Transform Infrared (FTIR)

Fourier Transform Infrared (FTIR) was used to analyze the functional group by using a FTIR spectrophotometer (IR-Prestige-21). The film was blended with KBr and pressed to yield a transparent and thin pellet. This pellet was placed on the sample holder, and scanned in the range of 500–4500 cm^{-1} in resolution of 2 m^{-1}, and 32 scans were taken for each sample.[10,41,44,50]

Mucoadhesive Force

The adhesive force of the film was evaluated using a Texture Analyzer (TXT 32), by attaching the film to the beef tongue mucosa. The film was placed on the tongue, and left in contact with the mucosa for 3 minutes. The top plate was then pulled at a constant speed of 0.05 mm/s until the film detach. The mucoadhesive force was recorded as the maximum force required to detach the film from the mucosa (in Nm).[41,51]

In-vitro Drug Release

In vitro drug release studies were done by dissolving the film in simulated saliva media. The simulated saliva (SS) media pH 6.8 was made by dissolving potassium phosphate monobasic 0.19 g/L, sodium chloride 8.00 g/L, sodium phosphate dibasic 2.38 g/L, and then placing it in a dialysis bag.[52] The bag was submerged in 500 mL of the same media at 37°C and stirred horizontally at 75 rpm. At fixed time intervals, 3 mL of sample was withdrawn, and the absorbance was measured with a UV-vis spectrometer at λmax of 316 nm. The release of the drug was examined on four mechanism models, namely: zero order, first order, Higuchi and Korsmeyer-Peppas.[53]

In-vivo Anti-RAS Evaluation

The study consists of design of study, size of sample, the inclusion and exclusion criteria, randomisation, blinding, measures of outcome, statistical analysis, the procedures and animal handling. This study obtained ethics approval from Universitas Padjadjaran Research Ethics Committee, Approval Code: 170/UN6.KEP/EC/2020, accordance to Guide for The Care and Use of Laboratory Animals Eighth Edition, and other applicable laws and regulations.

Study Design

In-vivo study created using the Experimental Design using a comparative study for the effect of two drugs on oral aphthous ulcer. Total 72 of rats blocked into 6 groups namely: CX, PM, α-.M, B, PC and NC, containing an equal number of rats. Each animal will be inducted with ketamine-xylazine, and an oral ulcer will be created on their tongue. The animal group will be treated with different treatments (including positive and negative control). On days 3, 5 and 7, 3 rats were euthanized by decapitation of the neck. The tongue specimen in each test group was cut off and followed by histopathological observation (Figure 2). Each measurement outcome will be analyzed by one-way ANOVA evaluation using Scheffe test in Statview to analyze the decrease of ulcer size and epithelization.

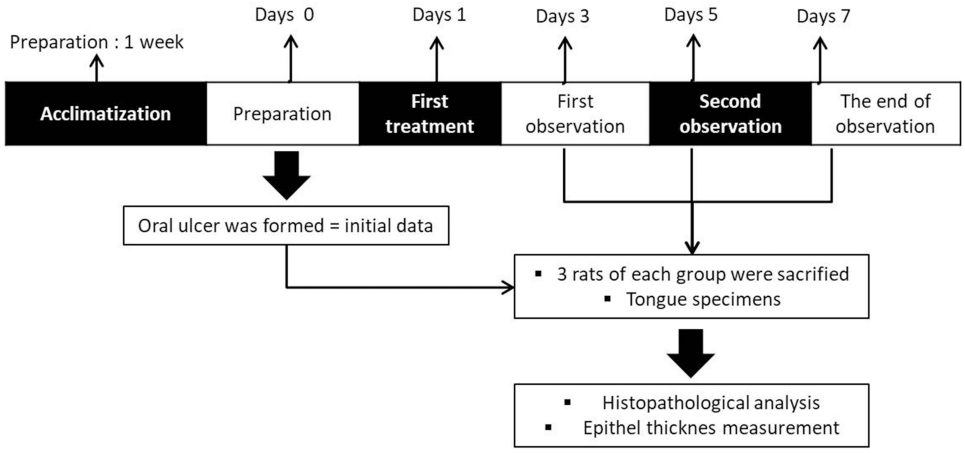

Figure 2 Experimental procedures for wound closure study in animal groups of α-M HMF, α-M/γ-CD PM HMF, and α-M/γ-CD CX HMF, compared to Positive Control (PC), Negative Control (NC) and HMF base.

Sample Size

The number rats were used in this study were 72 rats, divided into six groups: CX, PM, α-.M, B, PC and NC. These groups originally numbered 12 animals. The treatment received by the animals for each group, namely the CX group received α-M/γ-CD CX HMF, the PM group received α-M/γ-CD PM HMF, the α-.M group received α-M HMF, and the B group received HMF base. PC group received triamcinolone gel and NC group received no treatment. Because the tongue specimens of each group were taken at 0, 3, 5 and 7, each groups of rats should contains 12 rats.

Inclusion and Exclusion Criteria

The animals were included in the study if they underwent successful created an oral ulcer. The animals were excluded if the oral ulcer was not performed, or festering wound or if the animal died prematurely.

Randomization

Evaluation of anti-RAS was carried out using male Wistar strain rats aged 2–3 months and weighing around 200–250 grams, with a maximum age of 6 months. Prior to testing, the experimental animals were habituated for 7 days to adjust to the environment so as to minimize stress or pressure that would affect their physiological conditions were obtained from pharmacology Laboratory, Faculty of Medicine Universitas Padjadjaran Bandung and randomly divided into six groups (12 rats/group): the treatment, and control groups.

Blinding/Masking

For each animal, two different investigators were involved as: a first (YH) was responsible to create the oral ulcer and take the tongue specimens after treatment, whereas a second investigator (IS) assessed to the oral treatment.

Outcomes Measures

The following parameters were assessed: the level of inflammation is expressed as a score of 0–3, a score of 0 indicates low intensity of inflammatory cells, while 3 for high intensity of inflammatory cells;[7] and the thickness of epithelium was measured using the imageJ® application.[54]

Experimental Animals

Seventy-two animals were used: Wistar strain rats aged 2–3 months and weighing around 200–250 grams, with a maximum age of 6 months. Prior to testing, the experimental animals were habituated for 7 days to adjust to the

environment so as to minimize stress or pressure that would affect their physiological conditions. The rats giving the healthy and sufficient food, drink, and the environment (Figure 2).

Experimental Procedures

Evaluation of anti-RAS was carried out using male Wistar strain rats aged 2–3 months and weighing around 200–250 grams, with a maximum age of 6 months. Prior to testing, the experimental animals were habituated for 7 days to adjust to the environment so as to minimize stress or pressure that would affect their physiological conditions. At the beginning of the observation, histological data were collected before treatment as initial data.[7]

On the 1st day, the animals were administered ketamine i.p at a dosage of 75–100 mg/Kg, then a canker sore was created on the tongue using a filter paper previously soaked in glacial acetic acid. This filter paper is attached to the gums for about 25 seconds. The preparations were given every day at 8.00, with the distribution of the treatment groups as follows in Table 2.

On days 3, 5 and 7, three in each group were administered ketamine i.p at a dosage of 75–100 mg/Kg, and decapitation to get the tongue specimen for histopathological observation. Tongue specimens were taken and immersed in 10% formalin in phosphate buffer for 24–48 hours. Histopathological analysis was performed with haematoxylin and eosin (HE) staining. Analysis of the mucosal results of the biopsy was observed using a microscope with a magnification of 100x. The level of inflammation is expressed as a score of 0–3, a score of 0 indicates low intensity of inflammatory cells, while 3 for high intensity of inflammatory cells.[7]

Results

Physical and Mechanical Characteristic of HMF

The statistical analysis using the Statview application showed that there is no significant difference in the thickness of HMF base, α-M HMF, α-M/γ-CD PM HMF, or α-M/γ-CD CX HMF. The recommended film thickness is 12–100 μm with the maximum difference from the measurement should be less than 5%.[55–57]

The average thickness of α-M/γ-CD CX HMF preparations was 0.082±0.01 mm, while for HMF, α-M HMF, and α-M/γ-CD PM HMF preparations, 0.09±0.014 mm, 0.112±0.014 mm, and 0.109±0.007 mm respectively (Table 3). The α-M

Table 2 Treatment of Animal Test

No	Group	Treatment
1.	Negative Control (NC)	Induction with Ketamine-xylazine 75–100 mg/Kg i.p. RAS ulcer created
2.	Positive Control (PC)	Induction with Ketamine-xylazine 75–100 mg/Kg i.p RAS ulcer created Given 1% Triamcinolone
3.	Base (HMF)	Induction with Ketamine-xylazine 75–100 mg/Kg i.p RAS ulcer created Given HMF
4.	α-M HMF	Induction with Ketamine-xylazine 75–100 mg/Kg i.p RAS ulcer created Given α-M HMF
5.	α-M/γ-CD PM HMF	Induction with Ketamine-xylazine 75–100 mg/Kg i.p RAS ulcer created Given α-M/γ-CD PM HMF
6.	α-M/γ-CD CX HMF	Induction with Ketamine-xylazine 75–100 mg/Kg i.p RAS ulcer created Given α-M/γ-CD CX HMF

Table 3 Physical and Mechanical Properties

HMF	Thickness (mm)	pH	Tensile Strength (mPa)	Elongation at Break (%)
HMF	0.09±0.02	6.12±0.02	25.00±1.66	11.70±3.75
α-M HMF	0.11±0.02	6.02±0.02*	5.84±1.90	67.67±15.31
α-M/γ-CD PM HMF	0.12±0.01	6.07±0.01*	4.09±0.39	93.67±6.51
α-M/γ-CD CX HMF	0.08±0.01	6.16±0.01	4.63 ±2.18*†	118.00.±23.07*†

Notes: The results are expressed as the mean ± SD (n = 3). †$p < 0.0001$ and *$p < 0.05$ compared to HMF.

HMF and α-M/γ-CD PM HMF preparations were slightly thicker than this range due to the insoluble α-M powder, while the α-M/γ-CD CX HMF and HMF base fulfilled the thickness recommended for the film, which is 12–100 μm, because all of its components are completely dissolved in the film base.[56,57]

The pH of the film preparations was nearly uniform, and all were above 6.00, which is within the normal salivary pH range of 6–7. This is an important factor to consider since preparations with a pH below 5.5 can lead to demineralization of dental tissue. Therefore, these film preparations meet the requirements for safety and can be used without causing harm to dental tissue. A statistically significant difference was observed between α-M/γ-CD CX HMF, α-M HMF (†$p < 0.0001$) as well as α-M/γ-CD PM HMF (*$p<0.05$), with the pH 6.16±0.01 while the HMF base itself has a pH of 6.12 ±0.02 (Table 3). Both HMF and α-M/γ-CD CX HMF bases do not pose an irritation risk because they have a pH of more than 6.1 and meet the salivary pH requirements in the oral cavity.[58,59]

Tensile strength (TS) is the maximum stress that a film can withstand before breaking expressed in (N/cm^2). The recommended tensile strength for film preparations is more than 1 N/mm^2.[60–62]

The tensile strength of a film is influenced by the strong cross-links in the polymer. However, the addition of an active substance such as a drug molecule can decrease the tensile strength of the film base. Prior to adding the drug molecule, the film base had a tensile strength of 25±1.664 N/cm^2. When the active substance (α-M, α-M/γ-CD PM, or α-M/γ-CD CX) was added, the tensile strength decreased to 5.843±1.895, 4.087±0.393, and 4.630±2.178 N/cm^2, respectively (Table 3).

This happens because the cyclodextrin molecules from the physical mixtures α-M/γ-CD PM and α-M/γ-CD CX have hydrophilic properties, thereby reducing the tensile strength. The addition of active substances such as aloe vera decreases the tensile strength due to the hydrophilic nature of aloe vera.[41] A film preparation meets the requirements if it has an elongation break of more than 10%.[38–40] Elongation at break of α-M/γ-CD CX preparations, 118±23.06%, was the highest among other films, due to the entrapment of active substances from water-soluble complexes which reduced interactions between polymer chains and increased the mobility of these polymers, resulting in tensile strength decreased and elongation at break increased.[41,42] The value of tensile strength is inversely related to elongation at break, while high tensile strength will reduce the elasticity of a preparation.[41,63]

Swelling index measurements were performed to analyze the ability of the preparation to absorb the exudate from the wound. This is related to the absorption of fluid from the wound.[41,64] The swelling index of α-M/γ-CD CX HMF increased by about 1.53 times compared to HMF without active substance. In contrast to α-M HMF, which showed a reduced swelling index of around 23.59% compared to HMF, the swelling index of α-M/γ-CD CX PM HMF is nearly identical to that of HMF (Figure 3).

The porous network structure of HMF films results in a high degree of swelling and wetting time (Figure 4). The WVTR value is related to the porosity of the film.[65] The WVTR value can vary by the structural porosity of a film matrix. Therefore, WVTR measurements were carried out to determine the rate of water absorption into the film matrix, and scanning electron microscopy (SEM) was used to observe the surface and microstructure of the film.[44,49]

Furthermore, to examine the release mechanism of drug in the preparation, a curve fitting was carried out for each release model, namely the zero-order, first-order, Higuchi, and Korsmeyer-Peppas models. The release constant and R^2 value of each film were calculated, and then a curve fitting was done to determine the release mechanism of the drug. The

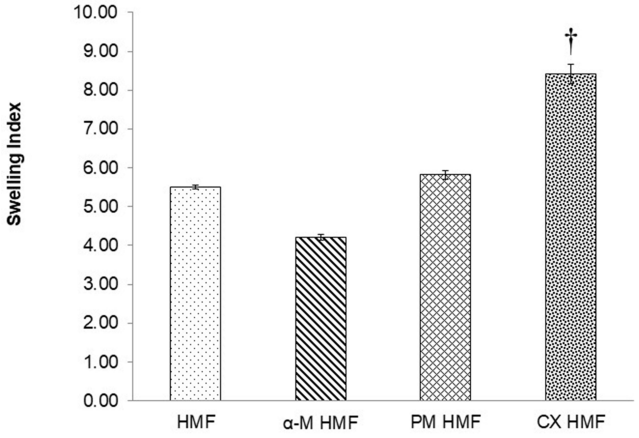

Figure 3 Swelling index of HMF, α-M HMF, α-M/γ-CD PM HMF, and α-M/γ-CD CX HMF. Each value represents the mean ± SD of three experiments. †p<0.0001, compared to HMF, α-M HMF and α-M/γ-CD PM HMF.

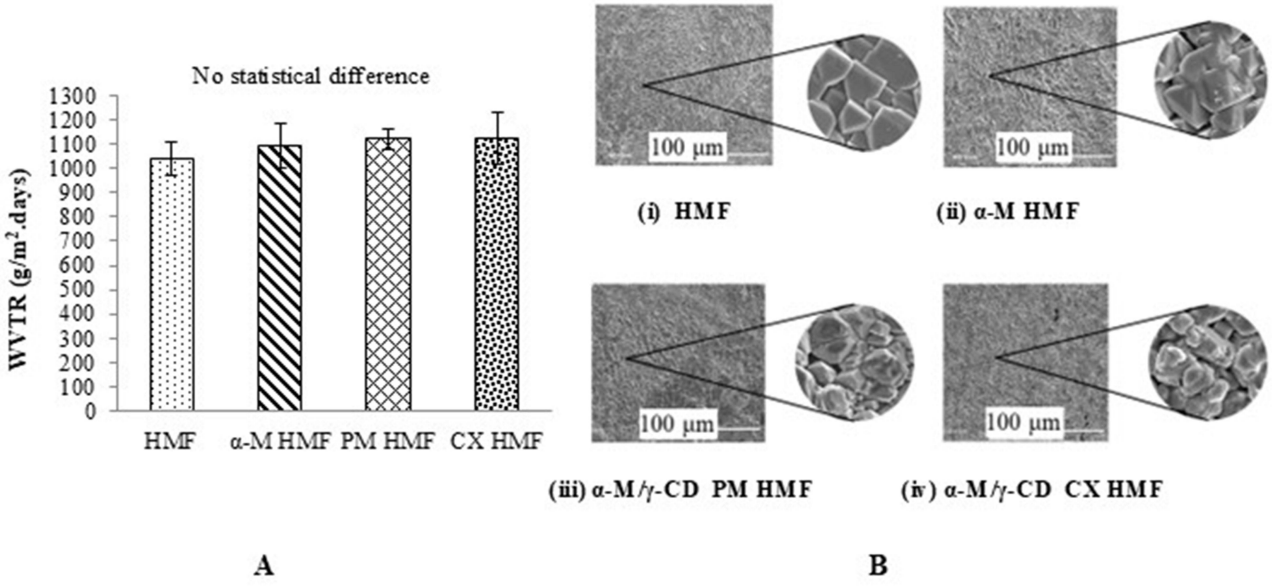

Figure 4 Water Vapor Transmission Rate/WVTR (**A**) and morphology (**B**) of HMF (i), α-M HMF (ii), α-M/γ-CD PM HMF (iii), dan α-M/γ-CD CX HMF (iv) in magnification 3000x. Each value represents the mean ± SD of three experiments.

discharge constants (K), both at zero order (K_0), first order (K_1), Higuchi (KH), and Korsmeyer-Peppas (KP), as well as the R^2 value of each HMF, can be seen in Table 4.

Curve fitting is carried out using a value of "n", which is 0.43, namely for films that have the ability to swell. The results of the curve fitting show that drug release follows the Higuchi and Korsmeyer-Peppas model based on the R^2

Table 4 Fitting Curve from in-vitro Drug Release of HMF, α-M/γ-CD PM, and α-M/γ-CD CX

Formulation	Zero Order		First Order		Higuchi		Korsmeyer-Peppas	
	K_0	R^2	K_1	R^2	K_H	R^2	K_P	R^2
α-M HMF	0.12±0.01	0.2913	0.02±0.02	0.7745	1.16±0.44	0.9211	1.61±0.66	0.9289
α-M/γ- CD PM	0.06±0.03	0.7548	0.02±0.01	0.8410	0.64±0.48	0.8096	0.91±0.69	0.8093
α-M/γ-CD CX	0.35±0.07	0.5623	0.04±0.03	0.6090	3.54±1.52	0.93	4.93±2.27	0.9342

Table 5 Mucoadhesive Time and Mucoadhesive Force

Formulation	Mucoadhesive Time (Minutes)	Mucoadhesive Force (Gforce)
HMF	50.43±1.91	814.11±12.10
α-M HMF	21.22±1.85	115.23±0.08
α-M/γ-CD PM HMF	23.44±3.97	73.80±0.11
α-M/γ-CD CX HMF	49.16±3.28†	1042.76±0.24*†

Notes: The results are expressed as the mean ± SD (n = 3). †$p < 0.0001$ and *$p < 0.05$ compared to HMF.

value in each preparation. In this model, the drug release process does not depend on time but on the geometrical structure of the constituent polymers, namely the polymer matrix making up the film base. Drug release occurs due to the swelling ability of the film-forming polymer, namely sodium alginate-chitosan.[66–68]

In-vitro Mucoadhesive Test

The mucoadhesive study of each film includes the mucoadhesive time and force. The mucoadhesive time of HMF, α-M HMF, α-M/γ-CD PM HMF, and α-M/γ-CD CX HMF were 50.42±2.57; 21.22±1.85; 23.44±3.97; and 49.16±3.28 minutes, respectively. Meanwhile, the mucoadhesive force was 814.11±2.10; 115.23±0.08; 73.80±0.11; and 1042.76±0.24 g-force for HMF, α-M HMF, α-M/γ-CD PM HMF, and α-M/γ-CD CX HMF, respectively (Table 5). The mucoadhesive time of α-M/γ-CD CX HMF was significantly different from that of α-M HMF and α-M/γ-CD PM HMF (†$p<0.0001$). Meanwhile, the mucoadhesive force of α-M/γ-CD CX HMF was significantly different from that of HMF, α-M HMF, and α-M/γ-CD PM HMF (†$p<0.0001$). This is because the swelling and adhesive ability of a polymer affect the adhesive time of a film preparation. Chitosan, as the cationic polymer has the strong interaction with the combination of polymers will increase the mechanical bond between its positive charge with the negative charge of sialic acid in the mucosal surface.[63,65]

In-vitro Drug Release

To analyze the mechanism of α-M release from HMF, a drug release test was carried out in simulated saliva media. An exponent value of $n = 0.5$ indicates Fickian diffusion, $0.5 < n < 1$ indicates the anomalous transport mechanism, and $n = 1$ indicates Case II transport.[66] The drug release was affected by the combination of polymer that used as a film base. Alginate has increased the swelling ability resulting the prolong relesae of α-M from HMF.[69] In addition, the entrapment of α-M in γ-CD as in α-M/γ-CD CX also modified the release of α-M from the cavity to enter the wound.

In the 5th minute of the drug release test in simulated saliva media, the percentage of α-M released from α-M HMF, α-M/γ-CD PM HMF, and α-M/γ-CD CX HMF was only 0.68±0.01%, 0.17±0.03%, and 2.28±0.05%, respectively. At the end of the test, the release of the active substance from α-M HMF was 26.144±0.20%, while for α-M/γ-CD PM HMF and α-M/γ-CD CX HMF, it was 22.53±0.09% and 80.34±0.32%, respectively. This indicates an increase of about three times compared to α-M HMF. This difference is attributed to the cyclodextrin in the α-M/γ-CD CX complex, which acts as a vesicle and increases the solubility of α-M in water (Figure 5).

In-vivo Anti-RAS Evaluation

Wound Closure

Wound closure studies were conducted to analyze the level of wound healing in the mucosa. The healing process is characterized by a decrease in the area of the wound over time. If the difference between the initial measurement of the wound and the measurement taken on a later day of observation is greater, it suggests that the wound healing rate is improving.[70,71] Figure 6 is a picture of the tongue during the test.

The wound area of each tongue was measured using the imageJ© application to obtain an accurate area, which was crucial as the shape of the wound is not perfectly round, which was challenging to measure. The uneven healing process from all sides of the wound circle contributed to this difficulty.

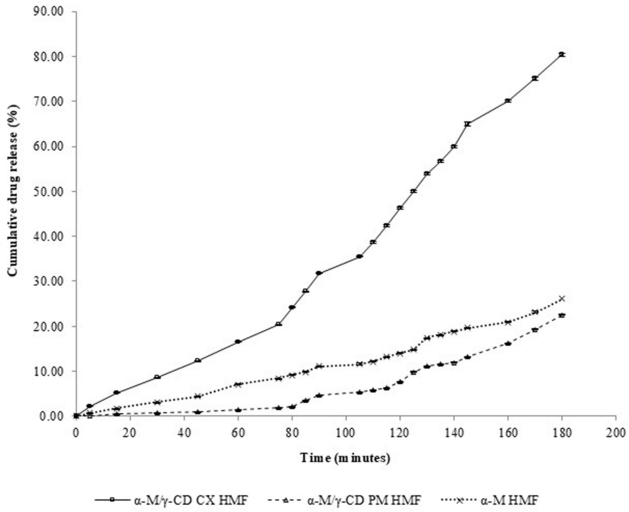

Figure 5 In-vitro drug release of α-M HMF, α-M/γ-CD PM HMF, and α-M/γ-CD CX HMF. The results are expressed as the mean ± SD (n = 3).

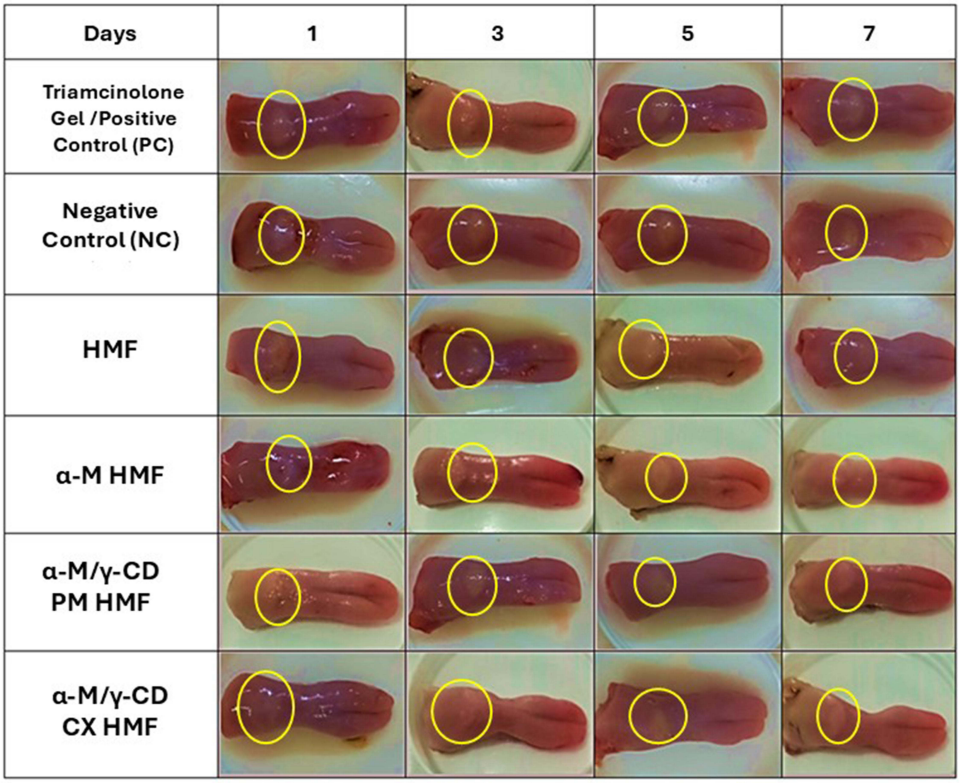

Figure 6 Wound closure study in animal groups of α-M HMF, α-M/γ-CD PM HMF, and α-M/γ-CD CX HMF.

Histopathological Analysis

Histopathological analysis was carried out by analyzing the part of the tongue of the rat affected by the lesion. Tongue specimens were obtained and preserved in 10% phosphate-buffered formalin. Tissues were blocked with paraffin and sectioned into slices of 3–5 μm thickness using a microtome. Slices were subjected to Haematoxylin-Eosin (HE) staining

to prepare them for analysis. The prepared tissue sections were analyzed using a microscope with a magnification of 40x to determine several parameters, including the comparison and formation of the epithelial lining (Figure 7).

In histopathological analysis, staining was performed using hematoxylin and eosin (HE), with the aim of differentiating between cells in the tissue. Hematoxylin is the most commonly used type of core dye derived from logwood tree extract, which imparts a purple color to the tissue. Eosin dye is acidic and negatively charged, giving a red or pink color when it binds to alkaline structures in cells, so it is often used to stain the cytoplasm. The combination of these two dyes allows the recognition of certain tissue components differently and can stain with different levels or degrees of color resulting in different color intensities.[72]

On day 7, α-M/γ-CD CX HMF group showed the difference of thickness compared to α-M HMF, α-M/γ-CD PM HMF, and PC, and also to NC and HMF ($^{\dagger}p < 0.0001$). At the end of the test, the epithelial thickness for the test animal

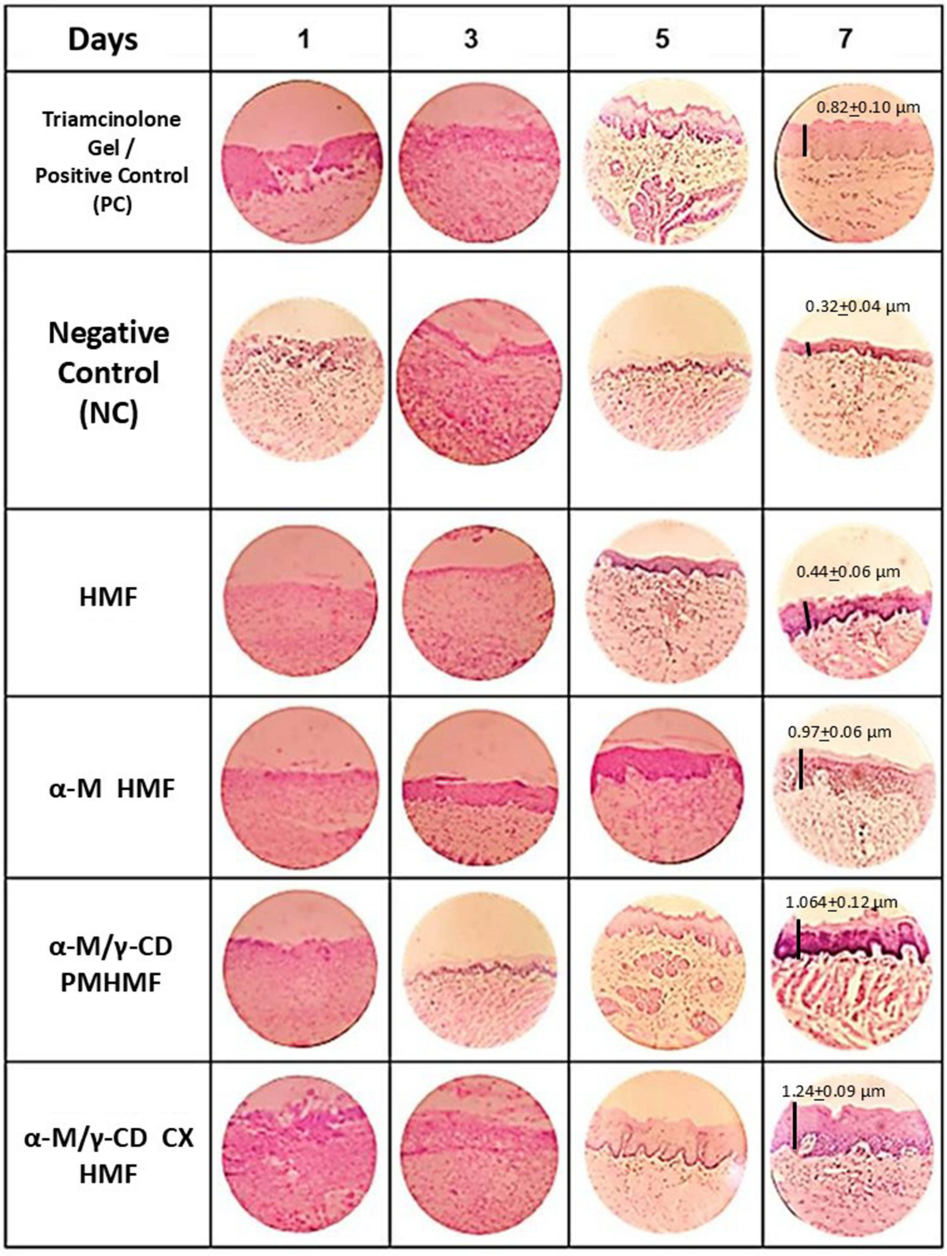

Figure 7 Epithelial formation in days of 1 to 7 in animals groups were given HMF, α-M HMF, α-M/γ-CD PM HMF, and α-M/γ-CD CX HMF.

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group that was given α-M/γ-CD CX HMF was 1.24±0.09 μm, higher than the group that was treated with α-M HMF, α-M/γ-CD PM HMF, and PC, respectively 0.97±0.06 μm, 1.064±0.12 μm, and 0.82±0.10 μm.

In addition to measuring the thickness of the epithelium, further analysis of the level of inflammation was carried out by measuring the intensity of inflammatory cells (neutrophils) as shown in Figure 7, to determine the decrease in the level of inflammation in the wound.

At the end of the test, there was a significant difference of α-M/γ-CD CX HMF group compared to α-M HMF, α-M/γ-CD PM HMF, and PC (*$p < 0.05$), meanwhile †$p < 0.0001$, compared to NC and HMF base (Figure 8). The measurement results were then analyzed using the Statview application.

The mechanism of α-M as an anti-inflammatory is reported to be through its inhibitory effect on NO, IL-β and TNF-α as well as selective blocking of NFkB and COX-2 together with iNOS blocking. Additionally, it has been reported that the anti-inflammatory effect of α-M also occurs through the blockade of NFkB translocation from the nucleus into the cytoplasm as well as inhibition of neutrophils.[73]

Discussion

Although α-M was dissolved in ethanol, the α-M HMF still revealed a non-homogenous film because of its poor solubility in water, and become to solidify when ethanol was evaporated. One of the method to improve the solubility in aqueous is the use of cyclodextrin. Cyclodextrins are classified based on the number of their constituent units, namely α-CD ($C_{36}H_{60}O_{30}$, BM: 972), β-CD ($C_{42}H_{70}O_{35}$, BM: 1135), and γ-CD ($C_{48}H_{80}O_{40}$, BM: 1297), each consisting of 6, 7, and 8 units of glucose pyranose molecules, respectively. γ-CD was used in this research because it has the biggest diameter among all cyclodextrin. α-CD, β-CD, and γ-CD have diameter of 4.7–5.3 Å; 6.0–6.5, and 7.5–8.3, respectively.[74] The molecular docking study of α-M with α-CD, β-CD, and γ-CD revealed that γ-CD was able to entrapped α-M in the inner cavity of γ-CD, because the formation of this complex has the lowest Gibbs energy.[1] To increase the solubility of α-M in water, the complex of α-M/γ-CD CX was prepared. This complex is completely soluble in HMF base resulting in homogenous HMF (Figure 9).

Ideally, a wound dressing should have a WVTR in the range of 2000–2500 $g.m^2$/day. However, achieving this value in practice can be difficult, and the industry has set different WVTR ranges for wound dressings. For example, some require a range between 90 and 3350 $g.m^2$/day, while others require a range between 480 and 3452 $g.m^2$/day.[73] In the current study, the WVTR values of HMF, α-M HMF, α-M/γ-CD PM HMF, and α-M/γ-CD CX HMF preparations were found to be 1037.054±67.001, 1094,363±90,546, 1119,403±40,718, and 1125.248±106.784 $g.m^2$/day respectively. Meanwhile, the WVTR of these four films was not significantly different (p=0.05), they do fall within the industry-specified range. This is crucial for ensuring that the film adheres quickly to the mucosal surface and maintains moisture on the wound surface in the oral cavity.[73]

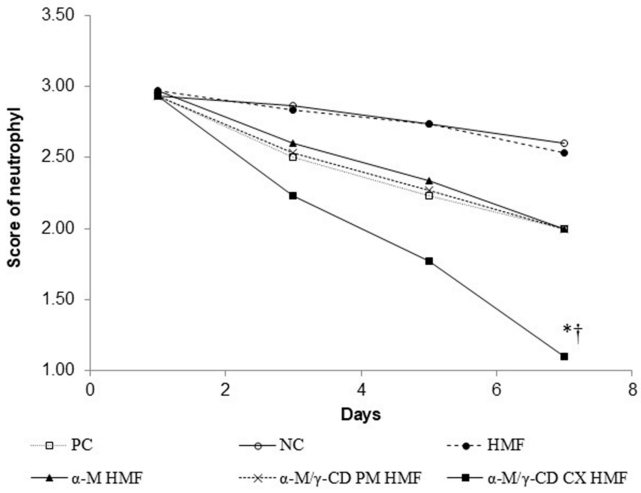

Figure 8 Scoring of inflammation on days of 3, 5 and 7. The results are expressed as the mean ± SD (n = 3). †$p < 0.0001$ and *$p < 0.05$ compared to α-M HMF, α-M/γ-CD PM HMF, and PC. †$p < 0.0001$, compared to NC and HMF.

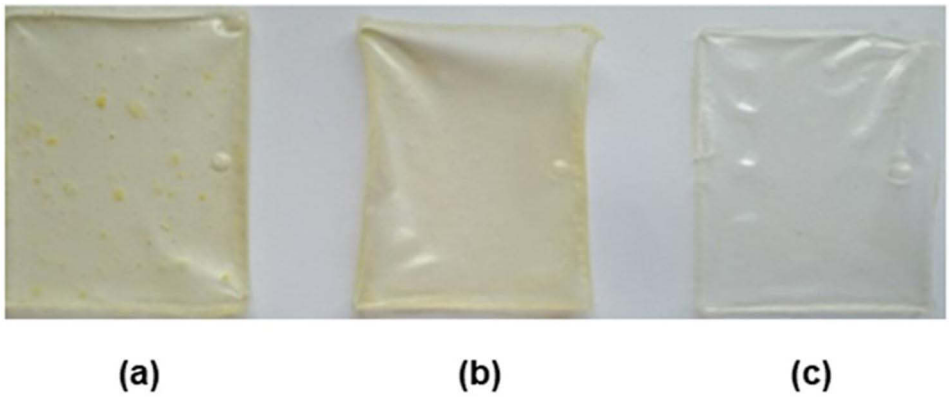

Figure 9 The appearance of α-M HMF (**a**), α-M/γ-CD PM HMF (**b**), and α-M/γ-CD CX HMF (**c**).

The combination of polymers will increase the mechanical bond between the polymer chains and the mucosa.[65] In this preparation, the combination of alginate polymer and chitosan increases the mechanical bond between the polymer and the mucosa,[41] due to hydrogen or ionic interactions between the positive charged on amino groups of chitosan with the negatively charged in mucus on the surface of the epithelium provided by sialic acid.[63]

A higher swelling index will significantly increase the contact area between the surface and the mucosa, facilitating a stronger interaction with the polymer in the preparation through hydrogen bonding. When the film is applied to the mucosal surface, it quickly becomes moist, absorbs liquid from the mucosa, adheres to it, and subsequently expands. As the film expands, it facilitates the release of the active substance, effectively delivering its therapeutic properties once it adheres to the wound.

Generally, porous chitosan structures exhibit a longer adhesion time than porous carboxymethyl cellulose (CMC) structures (Figure 3B). This is due to the cationic charge of chitosan. The amino groups in chitosan chain have positively charged, which interacts with the negative charged of sialic acid residues, and sulphate groups in the mucosa, which are abundant in the mucosal layer. As a result, this electrostatic interaction causes longer mucoadhesion times and higher mucoadhesion forces.[51]

Mucoadhesive polymers play a crucial role in promoting the healing process of recurrent aphthous stomatitis (RAS) by effectively covering oral wounds. This protective barrier created by the polymers helps minimize the severity of the wounds and inhibits bacterial proliferation. The results obtained demonstrate that α-M/γ-CD CX HMF preparations have the ability to extend the residence time of drug on the mucosal surface. This extended residence time enhances the therapeutic efficacy of the formulation by allowing sustained drug release and absorption. The combination of sodium alginate with chitosan is able to improve the characteristics of the sodium alginate film which disintegrates immediately in saliva.[41]

The salivary fluid, which usually contributes to the saliva washout effect, can facilitate the swelling of the film matrix due to the rapid interaction between sodium alginate and salivary fluid. Moreover, the incorporation of chitosan serves multiple purposes. Firstly, it participates in the formation of cross-links during the film preparation, which enhances the overall stability and integrity of the film. This cross-linking mechanism further prevents the rapid disintegration of the film upon exposure to saliva, allowing it to adhere to the surface of the oral mucosa for an extended period.[41]

Furthermore, to examine the release mechanism of drug in the preparation, a curve fitting was carried out for each release model, namely the zero-order, first-order, Higuchi, and Korsmeyer-Peppas models. The release constant and R^2 value of each film were calculated, and then a curve fitting was done to determine the release mechanism of the drug. The discharge constants (K), both at zero order (K_0), first order (K_1), Higuchi (KH), and Korsmeyer-Peppas (KP), as well as the R^2 value of each HMF, can be seen in Table 4.

Curve fitting is carried out using a value of "n", which is 0.43, namely for films that have the ability to swell. The results of the curve fitting show that drug release follows the Higuchi and Korsmeyer-Peppas model based on the R^2 value in each preparation. In this model, the drug release process does not depend on time but on the geometrical

structure of the constituent polymers, namely the polymer matrix making up the film base. Drug release occurs due to the swelling ability of the film-forming polymer, namely sodium alginate-chitosan.[66–68]

The release of the drug from the sodium-alginate/chitosan film matrix occurs gradually, starting from the outermost matrix and progressing towards the inner part of the film matrix, accompanied by swelling, which further improves the release of the drug from the expanding film matrix. This release mechanism causes an increase in the concentration of the active substances on the oral mucosal surface for a longer period of time, thereby increasing the residence time of the drug on the mucosal surface. The preparation sticks with sufficient time to provide its activity as an anti-RAS in oral wounds. This happens because saliva washout effect can be avoided, as the saliva that penetrates the film preparation is used to expand the sodium-alginate/chitosan matrix so that the active substance that is embedded within the film matrix is released and dissolves in the oral mucosa. Alginate quickly absorbs salivary fluid, while chitosan increases the strength of the film, so it does not disintegrate quickly. The synergistic combination of these two polymers strengthens the bond with the mucosa, so that during the initial stages, the outer layer of the film interacts with the mucosa, which in turn initiates the release process starting from the outermost layer of the film. As time passes, the polymer matrix expands and develops, allowing the active material to be gradually released from the inner layers of the film.

The α-M/γ-CD CX complex formulated in HMF preparations on a sodium-alginate/chitosan basis resulted in an increased anti-RAS activity due to the presence of a combination matrix of these two polymers which increases the residence time of the drug on the oral mucosa, therefore α-M has sufficient time to adhere to the oral mucosa for a longer duration without being rinsed off by saliva. The water-soluble α-M/γ-CD CX complex, which is embedded within this film matrix system, will help absorb the water that enters this preparation so that the film dissolves quickly compared to HMF containing α-M and a physical mixture of α-M/γ-CD PM, because both are difficult to dissolve in water so it takes a long time to penetrate the RAS wound, or will be rinsed before being absorbed from the surface of the oral mucosa, leading to lower activity of reducing RAS compared to films containing α-M/γ-CD complexes CX.

Wound healing activity has been provided by each HMF preparation, but for the untreated group (NC), the wound healing process is slow, while the α-M/γ-CD CX HMF treated group showed the fastest healing response, which is evidenced by forming scar tissue or epithelium covering the wound, which is attributed to the presence of soluble complex of α-M dispersed in the HMF preparation (Figure 5). Based on several studies reporting α-M has activity as an anti-inflammatory, antioxidant, and wound healing facilitating effect. The anti-inflammatory effect of mangosteen rind is strongly suspected through inhibition of inflammatory mediators such as COX-2, IL-1, IL-6 and NO 50. The anti-inflammatory activity of α-M is also mediated through its inhibitory effect on NO, IL-β and TNF-α as well as NFkB and COX-2 selectively together with iNOS blocking. It inhibits the translocation of NFkB from the nucleus to the cytoplasm, particularly at doses of 8 and 14 μg/mL. Furthermore, at doses of 14 and 25 μg/mL, it demonstrates substantial inhibition of neutrophils by 75% and 82%, respectively.[75]

α-M has a wide range of pharmacological activities, including anti bacteri, anti-inflammatory, wound healing, and anti-recurrent aphthous stomatitis. Because of its low water solubility, α-M has been complexed with γ-Cyclodextrin (γ-CD), resulting in increased solubility and improved compatibility in hydrogel formulations. The complex of α-M/γ-CD formulated into Hydrogel Mucoadhesive Film (HMF) preparations, resulting in the improvement of physical and mechanical properties of HMF. The improvement of mucoadhesive properties was due to the combination of two mucoadhesive polymers, namely alginate and chitosan, that were used as polymer base in this preparation. In addition, in-vivo RAS (Recurrent Aphthous Stomatitis) activity of the complex showed the higher RAS activity of the complexed α-M compared to uncomplexed α-M. This finding highlights the potential of the complex α-M/γ-CD in the treatment of oral aphthous ulcers.

Conclusion

α-Mangostin (α-M), which has poor solubility in water, has been made an inclusion complex with γ-cyclodextrin (γ-CD). This complex can be applied to alginate/chitosan hydrogel mucoadhesive film (HMF), resulting in the transparent yellowish film, while α-M HMF and α-M/γ-CD PM HMF are opaque. α-M/γ-CD CX HMF has better physical characteristics than α-M HMF and α-M/γ-CD PM HMF. HMF containing α-M/γ-CD CX has higher mucoadhesive

force and mucoadhesive time and prolonged retention time in the oral mucosa. The drug release of α-M/γ-CD CX HMF followed the Korsmeyer-Peppas Model with a total amount of drug released higher α-M HMF and α-M/γ-CD PM HMF. The modification of active compounds not only improved the physical characteristics of HMF, but also increased RAS activity. In-vivo evaluation in rats also revealed that of α-M/γ-CD CX HMF has shorten the time of healing, because of repair of tissue regeneration faster than α-M intact (α-M HMF) and the physical mixture (α-M/γ-CD PM HMF). The inclusion complex of α-M/γ-CD potentially used as an alternative to improve their formulation and enhance pharmacological uses of α-M. These findings highlight the potential use of α-M/γ-CD CX as an effective RAS agent in HMF.

Abbreviation

α-M, α-Mangostin; RAS, Recurrent Aphthous Stomatitis; γ-CD, γ-cyclodextrin; β-CD, β-Cyclodextrin; CX, complex; PM, Physical Mixture; HMF, Hydrogel Mucoadhesive Film; SEM, Scanning Electron Microscope; FTIR, Fourier Transform Infra-Red; XRD, X-Ray Diffractometry; Alg, Alginate; Chi, Chitosan; SS, Simulated Saliva; TS, Tensile strength; E, elongation at break; PBS, Phosphate Buffered Saline; WVTR, Water Vapour Transmission Rate; RH, Relative Humidity; PC, Positive Control; NC, Negative Control; HE, Haematoxylin & Eosin; NO, Nitrite Oxide; IL, interleukin; TNF-α, Tumor Necrosis Factor alpha (TNF-α); NFkB, Nuclear Factor Kappa-B; COX-2, Cyclooxygenase-2; iNOS, inducible Nitric Oxide Synthase; CMC, carboxymethyl cellulose.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest in this work.

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